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Exhibit D

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Clinical Protocol

Treatment of Advanced CNS Malignancies with the Recombinant Adenovirus H5.010RSVTK: A Phase I Trial

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I. Abstract

Primary CNS malignancies are responsible for approximately 12,000 deaths annually in the United States. There has been little change in the outcome for adults with malignant brain tumors over the past few decades, despite improvements in surgical techniques and advances in radiation therapy. These tumors are uniformly fatal one to two years after diagnosis. The morbidity and mortality of this disease arise from the effects of a locally invasive, non-metastasizing lesion. The patients may suffer from seizures, paralysis, incoordination, aphasia, confusion, memory loss, sensory deficits or visual loss, depending on the regions of the brain affected. In addition, they usually require large doses of corticosteroids early and late in their illness, and may experience disabling side effects of this treatment, such as edema, proximal myopathy, diabetes, fungal infections or deep vein thrombosis. Few patients in the older age group are able to work after the diagnosis. Most of the patients are incapable of self-care for several months before death. The localized transfer of new genes into cancer cells potentially permits the expression of proteins with specific biologic functions that may provide a means to alter the biology of tumor growth through a variety of mechanisms including increasing tumor immunogenicity, inducing the local expression of toxic agents, and sensitization of tumors to chemotherapeutic agents. Gene therapy with the transfer of the drug susceptibility gene Herpes virus thymidine kinase (HSV-TK) has shown promise in a number of animal models, including CNS tumors. This study will evaluate the use of adenovirus-mediated transfer of the HSV-TK gene into primary human brain tumors followed by systemic treatment with ganciclovir. The goals of this phase I study are to evaluate the overall safety and efficacy of this treatment and to gain insight into the parameters that may limit the general applicability of this approach. In this phase I study, patients with recurrent gliomas will receive stereotactic-guided injections of the virus into the brain tumor, followed by intravenous ganciclovir for 14 days. Patients eligible to undergo a palliative debulking procedure will receive the same treatment followed by resection on day 7. At the time of resection a second dose of virus will be administered intra-operatively into the residual, unresectable portion of the tumor, and intravenous ganciclovir will be continued for additional 14 days. Tissue removed at the time of resection will be analyzed for evidence of adenovirus infection, thymidine kinase expression and signs of inflammation. The size and metabolic activity of all tumors will be followed by volumetric MRI scans and Positron Emission Tomography Scans, respectively. Patients will be enrolled in groups of three, with each group receiving successively larger doses of adenovirus. This study will quantify the toxicity of this therapy, and provide evidence as to the duration of transgene expression and virus induced inflammation.

II. Background

II.A. Clinical Aspects of Malignant Brain Tumors

Malignant brain tumors are diagnosed in approximately 8,000 adults Americans yearly, and account for about 2.5 percent of deaths from cancer [1]. In adults, the primary brain tumors are most often malignant gliomas. There are some less common types of primary malignant tumors, such as lymphomas or sarcomas, as well as benign tumors such as meningiomas. The gliomas are classified as astrocytoma (generally benign, usually found in children), anaplastic astrocytoma, and glioblastoma multiforme. Occasionally, mixed tumors of intermediate grade are seen, such as the oligodendroglioma with anaplastic astrocytoma. The cell of origin for these malignancies is the astrocyte, a supporting cell in the brain. Therefore, the tumors arise in white matter, and can be found in any location, although about 90 percent are in the cerebrum. The gliomas spread locally, compressing and infiltrating normal brain. Growth into ventricles or meninges is uncommon, and metastases through the blood stream or lymphatics is extremely rare [2]. Frontal lobe tumors may cross to the opposite side of the brain through the corpus callosum. However, the spread is contiguous and multifocal disease is not often seen. Autopsy studies show that the tumors usually remain localized throughout their course [3,4]. After surgery or radiation therapy, the recurrences are close to the original tumor bed.

The population affected by gliomas consists of all ages and races, and there is only a slight male predominance. Most of the patients are older than 50 years [5]. In the younger age group, anaplastic astrocytomas are more common, but in patients over age 50, most of the tumors are glioblastoma. There is no known cause or means of prevention. The disease is devastating, due to its debilitating neurologic consequences and rapid termination in death. No one survives glioblastoma; only a few patients are long-term survivors of anaplastic astrocytoma. The patients may suffer from seizures, paralysis, incoordination, aphasia, confusion, memory loss, sensory deficits or visual loss, depending on the regions of the brain affected. In addition, they usually require large doses of corticosteroids early and late in their illness, and may experience disabling side effects of this treatment, such as edema, proximal myopathy, diabetes, fungal infections or deep vein thrombosis. Few patients in the older age group are able to work after the diagnosis. Most of the patients are incapable of self-care for several months before death. The survival depends on the histologic type of the tumor, as well as the location in the brain and the functional ability of the patient (measured as the Karnofsky performance status). The median survival varies from 58 months for those with anaplastic astrocytoma who are less than 50 years old with normal mental status, to 18 months for those with glioblastoma less than 50 years old and normal Karnofsky status, to 9 months for those with glioblastoma older than 50 years and fairly good Karnofsky status. Unfortunately, the majority of patients fall into the last group, so that the median survival for all adults with malignant gliomas is 9–12 months [6,7].

As can be surmised from the survival data, treatment for this disease is largely ineffective. Surgery and radiation therapy are the primary modalities of treatment, but certain characteristics of the glioma render them quite resistant to cure. Surgical resection is a goal in treatment of any localized malignancy. Indeed, these tumors are often small compared to cancers elsewhere in the body (less than 5 cm in diameter at diagnosis), but they cannot be completely removed for several reasons: they lack a defined capsule or edge, they infiltrate into normal-appearing brain, and they may be located within or adjacent to critical brain regions that cannot be removed without permanently disabling the patient. Therefore, partial resection is performed in most cases. In some patients only a biopsy can be done safely due to the site of the tumor (Broca's area or the thalamus). Surgery is safer since the wide-spread use of corticosteroids, Mannitol, and intra-cranial pressure monitoring; and laser technique and ultrasound localization improve the outcome. It is generally believed that partial resection is beneficial, in that it creates some space into which the residual tumor can grow before causing pressure symptoms. Patients who are able to undergo resection appear to live longer than those whose tumors are only biopsied. Second and third resections are occasionally performed for recurrent tumors in patients with good performance status.

Radiation therapy is applied to all patients with malignant glioma, except when the neurologic condition is too poor to permit it. The tumors do show response to radiation in many cases, but high doses are necessary to achieve control. The normal brain around the tumor can generally tolerate no more than 60 cGy which is a dose below the curative level for glioma. Therefore, a number of techniques have been devised to maximize the tumor dose. Hyperfractionation allows the therapist to deliver higher tumor doses by taking advantage of the ability of normal brain tissue to repair sub-lethal radiation damage. However, there is little evidence that hyperfractionated radiation therapy results in longer survival, despite many trials [7].

Radiation-sensitizing drugs such as Misonidazole and BUdR have been used, with little improvement [7]. Interstitial radiation with or without hyperthermia permits the delivery of very high doses directly into the tumor and spares much of the normal brain. This technique does seem to result in better survival, and even in the eradication of gliomas in some cases. However, it results in radiation necrosis of brain in about half the patients, necessitating another operation [8]. This technique is applicable to only about one-third of patients, since the tumor must be in an anatomically accessible location.

Other approaches to glioma treatment have included chemotherapy, either as an adjuvant to radiation [9], or at the time of relapse [10], and immunotherapy, with interferons [11], intra-tumor LAK cell instillation [12], radio-labeled monoclonal antibodies or other techniques [13]. None of these methods is curative. Adjuvant chemotherapy is helpful in a minority of patients, but probably does prolong median survival at least for anaplastic astrocytoma. For the bulk of patients (mostly glioblastoma) adjuvant chemotherapy increases median survival only from 9.4 months to 12 months [14].

At the time of relapse or recurrence of the tumor, the patient's survival is rarely more than six months. If the patient's performance status and the site of the tumor permit it, a second resection is usually performed. Chemotherapy given at the time of relapse controls the tumor or provides a remission in only about 30 percent of patients. The poor results from chemotherapy may be partly due to the inability of most chemotherapy drugs to cross the intact blood–brain barrier at the edges of the tumor [15]. Areas of necrosis and areas with poor blood supply within the tumor are less accessible to chemotherapy. Additionally, the tremendous heterogeneity of these tumors results in chemotherapy resistance in many of the cells. Attempts have been made to improve delivery of chemotherapy into brain tumors by giving the drugs directly into the carotid artery, by temporarily opening the blood brain barrier with hypertonic agents such as Mannitol [16], and by implantation of chemotherapy containing polymers directly into the brain tumors [17]. There have been significant complications of some of these methods, without consistent improvement in outcome. High dose chemotherapy with bone marrow rescue has also been utilized, but again the toxicity has been high and the results poor [18].

In summary, despite a large body of clinical research in the treatment of malignant gliomas, there has been little overall improvement in the outcome for these patients.

II.B. Preclinical Studies

II.B.1 The Use of HSV TK with Ganciclovir in Cancer Models

The failure of conventional brain tumor therapies stems from their inability to functionally distinguish neoplastic from normal cells. Despite considerable efforts, tumor specific molecu-

lar attributes that are suitable therapeutic targets have not been identified. Creation of an "artificial" difference in biochemical function is an attractive option [19]. Insertion of the herpes simplex thymidine kinase (HSV-TK) gene into malignant cells in conjunction with the systemic administration of ganciclovir (GCV) has become a prototypic gene therapy system for the selective destruction of cancer cells [19]. This section provides a general overview of the background experiments that have used HSV-TK as a "suicide gene" to selectively target cancer cells in experimental systems.

Many investigators have shown that the expression of the HSV-TK gene confers a negative selectable phenotype to cancer cells in vitro. Moolton [19] demonstrated acquired GCV sensitivity in a murine sarcoma cell line transduced with a retroviral vector that produces HSV-TK. The transduced sarcoma tumor cells were 200–1000 times more sensitive to GCV than control tumor cells. This finding has been reproduced in several rodent and human cancer model systems including lung cancer [20], mesothelioma [21,22], hepatocellular carcinoma [23], leukemia [24], melanoma [25] and CNS tumors [26–32] models. The efficacy of this approach varies significantly [24] and may be due to a variety of factors including promoter function, target cells studied, and efficiency of transduction.

Retroviral vectors were employed for much of the early experiments with HSV-TK. Several modifications have been introduced to overcome the difficulty of producing high titer retroviruses. Takamiya et al. [31] demonstrated that rat glioma cells co-infected with a retroviral TK vector and a wild-type (replication competent) vector were 300 fold more sensitive to the toxic effect of GCV, than those cells infected with TK vector alone. The co-infection permits the continued production of TK bearing virus and thus infection of neighboring tumor cells not transfected by the initial inoculation. This process in effect creates a retrovirus packaging cell line within the tumor. Takamiya et al. [31] subsequently improved upon this system by introducing a murine retrovirus packaging cell line directly into the tumor [30]. This approach has been studied in other CNS tumor models [29], in experimental hepatic metastases [23], and has become the basis of ongoing clinical trials for the treatment of brain tumors.

More recently, adenovirus vectors have been used for gene therapy of brain tumors. Chen et al. [32] demonstrated regression of experimental gliomas following in vivo adenovirus-mediated gene transfer and GCV treatment. The tumor deposits were not completely eliminated by this treatment, however. Tumor cells close to the injection site were more readily transduced than those distant, as judged by parallel marker gene transfer experiments. Furthermore, these more distant cells escaped GCV toxicity because of a diminished bystander effect attributed to a paucity of gap junctions in the rodent brain tumor cell line employed [32]. This can be potentially overcome in the clinical setting by more precise stereotactic treatment planning (aided by MRI and PET studies), and by multiple tumor injections.

The tumoricidal activity of the HSV-TK/GCV system is due to several factors. In dividing cells, the phosphorylated GCV inhibits DNA synthesis. This effect is not confined to cells that are directly transduced with HSV-TK, as neighboring cells are also affected. This phenomenon, which likely occurs as a result of several mechanisms, has been termed the "bystander effect" and has been observed in several tumor types including CNS tumors. Transfer of the phosphorylated GCV between cells, ("metabolic cooperation") via gap junctions has been proposed as a possible mechanism. Phagocytosis by neighboring cells of GCV phosphate containing apoptotic vesicles (from dying transduced cells) has also been proposed [33]. Immune-mediated processes may also account for significant killing of non-transduced cells. In one report [28], anti-tumor immunity was observed following TK mediated killing of experimental brain tumors. This protective immunity led to eradication of subsequent tumor cell inoculi when the animal was rechallenged at a remote site [28]. Whether the tumor immunity is TK dependent, or merely a manifestation of inherent tumor cell immunogenicity, has yet to be established in this rodent model.

These important findings have served to point out the limitation of this approach. Golumbek et al. [25] demonstrated that HSV-TK was unable to completely eliminate non-immunogenic tumor cells even when 100% of the introduced cells exhibited TK expression. They observed a delayed outgrowth of TK^+ tumor cells occurring many days after cessation of GCV. These cells were likely in G_0 arrest during the interval of GCV therapy and thus were able to escape the toxic effects of GCV metabolites. Alternatively, they were sequestered in an area of poor GCV penetration and thus received sublethal doses of the drug.

II.B.2 Toxicity of Ganciclovir

Ganciclovir is a synthetic nucleoside analog that is approved for the treatment of CMV retinitis in patients with acquired immunodeficiency syndrome and for the prevention of CMV infection in bone marrow transplant recipients. In addition, it is used to treat CMV infections in transplant patients. Extensive clinical experience exists in the use of this drug. It is distributed widely to all tissues including the CSF and brain parenchyma. Ganciclovir has been shown to exhibit several toxic side effects in humans. Mammalian cell proliferation (in particular bone marrow) is affected (in vitro) at high concentrations (10 to 60 mg/ml). Granulocytopenia and thrombocytopenia have been observed in patients treated with ganciclovir. Renal impairment has been attributed to ganciclovir, especially when used in combination with other nephrotoxic drugs. Seizures have been observed when this drug was administered in combination with imipenem-cilastatin. It has been shown to be carcinogenic, teratogenic and caused aspermatogenesis in animal studies. Headache and confusion have been observed in transplant patients at frequencies of 17% and 6%, respectively. Other frequent adverse effects include anemia, fever, rash and abnormal liver function tests. Each of these occurred in approximately 2% of patients.

II.B.3 The Clinical Spectrum of Wild-Type Adenovirus Infections of the CNS

Adenovirus CNS infection is sporadic and uncommon, although its true incidence can not be estimated from case reports. Undoubtedly many cases are neither diagnosed (aseptic meningitis) nor reported. From the reports available adenovirus meningoencephalitis is usually self-limited and the clinical outcomes governed by the presence of underlying disease in the affected patients. We anticipate that the use of a

replication defective adenovirus will substantially limit the spread of the virus and attenuate the pathologic consequences. This has been established more definitively in our ongoing toxicity studies.

II.B.4 Experimental Wild-Type Adenovirus Infections of the CNS in Primates

In the course of developing an adenoviral vaccine the CNS toxicity of wild-type adenovirus strains were studied. Rorke and colleagues [34,35] systematically studied the neurovirulence of several adenovirus serotypes (1, 2, 3, 4, 5, 7, 14, and 21) following direct injection of the virus into the CNS of rhesus monkeys. Focal injection of the virus into the CNS (up to 0.5 ml in to each thalamus, and up to 10^7 $TCID_{50}$/ml) elicited a spectrum of responses depending on the serotype of the virus. They demonstrated that, independent of serotype, viral antigen could be detected in the ependyma, choroid plexus and leptomeninges within 72 hours of injection into the thalamus [34]. This indicates that the wild-type virus is potentially capable of spreading within the substance of the brain. These experiments were done without the use of a stereotactic device (personal communication from Dr. Lucy Rorke, University of Pennsylvania) and that the localization of the injection site was confirmed retrospectively by the finding of an injection site granuloma at necropsy. Animals injected with various serotypes were observed for eighteen days and then necropsied. The histologic findings ranged from no significant pathologic findings to lymphocytic infiltration of the leptomeninges and choroid plexus, with ependymitis and focal neuronal destruction. Interestingly, this resulted in no overt symptoms of CNS disease. The authors note that "clinical observation disclosed no signs or symptoms among the monkeys with evidence of inflammatory lesions that distinguished them from those without such lesions" [34]. Serotype 5 adenovirus produced lesions of mild to moderate intensity. The results were very consistent within each serotype and appeared to be "an all-or-none phenomenon" [34]. CNS virus titers decline over a 21 day period, suggesting that viral infection is a self-limited process. Furthermore, intranuclear inclusion bodies that are typically associated with adenoviral infections in permissive tissues are specifically absent in CNS. This finding is consistent with the relative lack of CNS tropism of adenovirus [34].

II.B.5 Recombinant Adenovirus-Mediated Gene Transfer in Murine CNS

Adenovirus-mediated gene transfer, unlike retrovirus gene transfer, has the potential advantage of transducing non-dividing cells. Brain tumor cells are heterogeneous with respect to the fraction in active cell cycle, yet this is unlikely to alter their transducibility. Those cells undergoing gene transfer while in G_0 will be rendered sensitive to GCV provided it is given over a long enough period of time and that transgene expression persists over this period. We and other authors [36–39] have demonstrated the effectiveness of adenovirus vectors in the CNS. Direct injection of adenovirus into the brain parenchyma results in efficient gene transfer followed by gradual decline in transgene expression [39], but could be detected up to 30 days post injection [36].

Preliminary data suggest that significant replication and distant spread of the virus do not take place. The liver and spleen are readily transduced by intravenously administered adenovirus. However, when Davidson et al. [39] injected recombinant adenovirus in the brains of rats, they were unable to identify virus in the liver or spleen (by X-gal staining). In addition, they were unable to isolate the virus from retro-orbital venous blood samples that drained the injected area. Local toxicity at the site of injection was observed. This was probably secondary to a reactive gliosis from the injection itself [34] (seen in sham injected control) and cytopathic effects in the neuronal cells exposed to a very high multiplicity of infection along the injection tract [37].

II.B.6 Adenovirus-Mediated Gene Transfer in Non-Human Primate CNS

As part of an investigation of hypoxanthine phosphoribosyl transferase (HPRT) gene therapy for the treatment of Lesch-Nyhan Syndrome, Davidson and colleagues have used adenovirus vectors to deliver the HPRT gene into the CNS of non-human primates [40]. The information obtained on adenovirus vector CNS toxicity is relevant to the current study. In these studies adenovirus H5.010RSV*rhprt* (Ad.RSVrHPRT) was injected (200 μl) into the CNS of rhesus monkeys at concentrations ranging from 10^9 particles/ml to 1.6×10^{11} particles/ml. There were no immediate postoperative complications and there were no signs of cerebritis, meningitis, or encephalitis. The animals groomed and fed normally and there were no obvious signs of impaired neurologic function. One animal was euthanized after one week (monkey #1). Lymphocytes and foamy macrophages were found within the injected areas, and the numbers decreased with increasing distance from the injection site. This is consistent with a localized area of inflammation. Monkey #2 was treated in the same manner as monkey #1 but was clinically followed. He remained well, and MRI and PET scans after one month revealed no abnormalities. In vitro tests of peripheral blood lymphocytes showed no evidence of a increased T cell response to adenovirus infected fibroblasts. Of the two other monkeys studied, both remain clinically well. One monkey #4, developed MRI changes at the injection site consistent with localized edema. This finding persisted for greater than one without an increase or decrease in size. Monkey #3 that was similarly treated at a lower dose developed no MRI changes over the same time period.

These studies suggest that recombinant adenovirus administered into the CNS of primates produces a localized dose dependent inflammatory response which is clinically inapparent. These studies suggest that the toxicity of intracranial adenovirus administration will not be of a magnitude that precludes its use in humans.

II.B.7 Adenovirus-Mediated TK Gene Transfer in Rats with CNS Tumors

Intracerebral gliomas were induced by injection of 10^5 9L gliosarcoma cells (5 μl) in the forebrain of Fischer rats. The location and size of the tumor were determined by MRI imaging on approximately day 16 post implantation. Using the MRI scan information, the glioma mass was injected with H5.010RSV*TK*. Treatment with ganciclovir (15 mg/kg) was initiated 24 hours

post virus injection and was repeated twice daily thereafter. The growth of the untreated glioma has been previously shown to be exponential over the entire growth period (B. Kim and B.L. Davidson, unpublished data). The tumor doubling time was determined before H5.010RSV*TK* administration and GCV treatment. Marked growth retardation during the treatment period was observed. All of the rats treated with Ad.RSVlacZ and GCV succumbed to their tumors by day 28. In contrast, the rats treated with H5.010RSV*TK* and GCV, showed a significant delayed in mortality, with 2/8 of the rats surviving to day 40.

II.B.8 Adenovirus-Mediated TK Gene Transfer in a Human Brain Tumor Xenograft Animal Model

In vitro Studies: Dr. Peter C. Phillips conducted a survey of primary human brain tumor cell lines, each of which is tumorigenic in athymic mice or rats. Human glioma cell lines included U251, U87, U373, WF, JHG31, and JHG10, and A172. Medulloblastoma cell lines included D324, D425, D283, and CHOP 707. All cell lines are continuous (i.e., passaged >70×) and are well characterized. Adenovirus transduction efficiency was evaluated by in vitro transfection of glioma or medulloblastoma cells with an adenovirus vector containing the β-galactosidase reporter gene (H5.010CMV*lacZ*). At infection ratios of 10 viral particle per cell, 48–70% of cells demonstrated nuclear staining. At higher infection ratios, 100 particles/cell, 100% of cells expressed β-galactosidase. No difference in vector transduction efficiency was detected between glioma and medulloblastoma cell lines.

The cytotoxicity of H5.010RSV*TK* adenovirus-transduced cells to GCV was evaluated by a clonogenic assay or MTT assay. LD50 values for GCV in the 4 medulloblastoma cell lines ranged from 5.5–16 nM. In contrast, cells transduced with a control adenovirus (H5.010CMV*lacZ* adenovirus) had LD50 values of 270–310 nM. Glioma cell lines ranged in sensitivity from 10–78 nM. One cell line (G10) had a mean LD50 value of 200 nM, probably reflecting the long doubling time (96 hrs) and low growth fraction of this tumor. A bystander effect to GCV was demonstrated in selected medulloblastoma (D324) and glioma (U251) cell lines. Cytotoxicity to bystander cells was maintained below a 20:1 ratio of uninfected to infected cells. Cell contact was not essential for the bystander effect as demonstrated by the cytotoxic effects of filtered conditioned media on non-transfected medulloblastoma cells.

Flank xenograft experiments were performed with human brain tumor cell lines in nude mice. The mice were injected with 50 μl of D324 medulloblastoma or U251 glioma tumor homogenate in the right flank on day 1 and the size of the tumor was measured daily. When the median tumor volume reached 0.4 cm^3 the tumors were injected with H5.010RSV*TK* adenovirus. In addition, one group of nude rats bearing medulloblastoma flank xenografts was allowed to grow until the median tumor volume reached 0.8 mm3 prior to H5.010RSV*TK* adenovirus injection. Anesthetized mice were injected slowly over 15 minutes with 1×10^{10} H5.010RSV*TK* viral particles in a 100 μl volume. Seventy-two hours after viral injection, the mice were treated with 15 mg/kg ganciclovir intraperitoneally, twice daily for 14 days. Control animals received an intratumoral saline injection (100 μl), followed 72-hours later by ganciclovir at the same dose and schedule. Tumor size was measured twice weekly thereafter and tumor volume was calculated as: volume = (width^2 × length)/2. All glioma flank xenografts had complete resolution of tumor within 50 days after the start of GCV.

Medulloblastoma response to GCV was dependent on tumor size at initial treatment. All tumors demonstrated complete volume regression. No late tumor recurrences were observed in this group. By contrast, relatively large tumors (i.e., median tumor volume > 0.8 cm^3) showed a significant growth delay but only 2 of 6 tumors demonstrated growth reduction.

II.B.9 Recombinant Adenovirus Vectors in Human Gene Therapy Trials

Recombinant adenoviruses are being employed in gene therapy trials at several institutions. Human clinical studies are underway to determine their efficacy and safety in treating cystic fibrosis. From the limited data available at this time, it appears that the vector itself is likely to induce some degree of inflammatory response that is independent of the transgene employed. Zabner and colleagues [41] observed mild inflammation in the nasal epithelium of patients treated by nasal application of an Ad2.CFTR vector. This was thought to be secondary to procedure-related trauma as similar nasal epithelial changes were seen in normal volunteers sham treated with saline rather than virus containing solutions. Intrabronchial administration of Ad.CFTR has been noted to cause pulmonary infiltrates in cystic fibrosis patients receiving greater than 10^9 pfu of virus. These changes are similar to inflammatory lesions induced in non-human primates in preclinical toxicity studies. Simon et al. [42] noted the development of a mononuclear cell inflammatory response in baboons who received 10^9 and 10^{10} pfu/ml. While not clinically apparent, this did result in radiographic changes on chest radiographs that were consistent with the alveolar wall damage and intra-alveolar edema [42].

III. Clinical Design

III.A Summary

Gene therapy with the transfer of the drug susceptibility gene Herpes virus thymidine kinase (HSV-TK) has shown promise in a number of animal models, including CNS tumors. Given the extremely poor prognosis of patients with recurrent brain tumors and the development of new gene transfer technology, we wish to undertake a study of HSV-TK gene transfer for the treatment of adult patients with glioma. This study will evaluate the use of adenovirus-mediated transfer of the HSV-TK gene into primary human brain tumors followed by systemic treatment with ganciclovir. The goals of this phase I study are to evaluate the overall safety and efficacy of this treatment and to gain insight into the parameters that may limit the general applicability of this approach.

Adult patients with glioma that have recurred despite the standard dose of radiation therapy will be considered for treatment if they: 1) present with tumors not adjacent to the chiasm or brainstem, and without subependymal spread, 2) exhibit minimal cerebral edema by MRI scan, and 3) have a performance status of at least 70% by the Karnofsky scale. Twenty four patients will be entered into one of two phase I trials. Twelve pa-

tients with tumors amenable to palliative resection (Phase IA study group), and twelve with surgically unresectable lesions (Phase IB study group) will be sought. Three patients from each group will be treated at each of four virus dose levels. The adenovirus will be stereotactically injected into multiple sites of the tumor. Two days later, GCV treatments will be initiated (intravenously, twice a day). MRI scans will be performed on the day of stereotactic injection, and on days 2, 7, and 28. The day 2 MRI study will detect any post-procedure bleeding and will serve as a baseline for subsequent studies. Minimal procedure induced inflammation is anticipated at this early time point. PET scans will be performed preoperatively and on days 14 and 28. Seven days postoperatively, the patients in group IA will again be taken to the operating room for tumor debulking. A second injection of virus into the tumor bed will be performed intraoperatively under direct visualization with a dose of virus equal to the patient's initial dose. GCV will be administered for another 14 days. Histologic and PCR studies will be performed on the resected tissue to assess the amount of viable tumor, the presence of transgene (HSV*tk*) and HSV*tk* protein and the degree of lymphocytic infiltration. The patients in the inoperable group (phase IB study group) will be treated for a total of 14 days with GCV, and then followed clinically and by MRI and PET scans for evidence of tumor response or toxicity. The phase I studies will provide information on the systemic toxicity associated with stereotactically and intraoperatively delivered recombinant virus expressing thymidine kinase. Additionally, by examining the resected tissue specimens of those patients undergoing palliative resection following gene therapy, we will gain valuable information concerning the duration of transgene expression and extent of tumor penetration by adenovirus.

III.B Study Design

III.B.1 Patient Eligibility

III.B.1.a Patient Selection

Eligible patients must be >18 years old and have a histologic diagnosis of malignant glioma (anaplastic astrocytoma or glioblastoma), which has recurred after primary treatment. The first treatment must have included radiation therapy with or without chemotherapy. Radiation should have been completed at least 2 months prior to this study. There must be MRI scan evidence of tumor growth after treatment. Since treatment of glioma often induces brain edema or necrosis of brain, which may be difficult to distinguish from tumor growth, it is required that the tumor area show enhancement on MRI and that there be a clear increase in the enhancing area over a period of time. Positron emission tomography (PET scan) will be used to help differentiate recurrent tumor from radiation necrosis. These techniques reflect metabolic activity in the brain, and generally demonstrate increased uptake of radionuclide in malignant tumor but decreased uptake in necrotic lesions. The aforementioned requirements should ensure that the patients selected for this study have recurrent disease despite standard therapy. Such patients have a median survival of 6 to 12 months, depending on their age and performance status. Therefore, this is an appropriate group for experimental therapy.

The other eligibility requirements will help to select patients who can tolerate the proposed protocol with minimal concerns regarding safety. The patients must have no serious uncontrolled medical problems (such as infections, diabetes, angina, congestive heart failure). They must have a Karnofsky performance status of 70% or greater, meaning that they are independent in self care. Their MRI scan must show no herniation or marked midline shift, so that they can undergo neurosurgery and injection of fluid into the brain. Solitary tumors are preferred. However, if the patient has multifocal glioma, the largest tumor must be in an area that can be surgically accessible for debulking. (Multifocal gliomas will offer an interesting opportunity to test the hypothesis that viral injection into one area of the brain will result in a "bystander effect" in distant regions.) The tumor for injection must not be adjacent to the optic chiasm or brain stem and must not be associated with subependymal spread. Patients will be recruited from all races and both genders will be equally represented.

Laboratory evaluations prior to entry will include routine hematology, coagulation profile, chemistry test, chest x-ray, and EKG, all of which should be within normal limits. Mild abnormalities of liver function tests (less than 2 times the upper limit of normal) will be acceptable if patients are taking anticonvulsants which often cause these aberrations. A pregnancy test will be required in women of child bearing potential and such patients will be instructed to use contraception during and for 3 months after the adenovirus study.

III.B.1.b Screening Evaluation

Eligibility will be established by a review of the patients medical records with attention to the dose of radiation received, and a review of their MRI scans for tumor size and location. Two to three weeks prior to the planned therapy written informed consent will be obtained from the patient. Screening will consist of complete physical and neurological examinations, evaluation of performance status, blood chemistry screen, complete blood counts, blood coagulation profile, anti-adenovirus antibodies testing, pregnancy test (when indicated), chest x-ray, and review of prior histologic specimens from the brain tumor.

III.B.2 Imaging Studies

Volumetric MRI scans and FDG/PET scans of the brain will be obtained to assess the pretreatment size and metabolic activity of the tumor. The major anatomic goals will be to define volume of enhancing tissue as measured on "T1-weighted" volumetric gradient-echo image sets obtained before and after administration of gadopentate dimeglumine contrast material [43]. The volume of edema detected on Fast Spin-Echo "T2-weighted" images will be determined. This will include portions of the neoplasm, white matter infiltrated by neoplastic cells and vasogenic edema located beyond the confines of the tumor. The presence and severity of mass effect will be evaluated using linear measurements on the T1- and T2 weighted images. The volumetric MRI scans will be superior to standard MRI scans because they will permit more accurate estimation of tumor volume, and shape. This technique will be helpful in determining the areas of the tumor to be injected and for cal-

culating the virus dose per unit volume in these irregularly shaped and inhomogeneous tumors.

The positron emission tomography technique with F-18 fluoro-deoxy glucose (FDG) is able to identify high grade tumors (high glucose metabolic rate) and differentiate necrosis (low glucose metabolic rate) from recurrent tumor more reliably than MRI scan [44,45]. Prior studies have demonstrated that the glucose metabolic rate falls prior to a clinical response to cancer treatment [46,47]. FDG-PET thus will give us an independent method for following tumor response.

III.B.3 Clinical Trial

The patients will be admitted to the neurosurgery service and evaluated to establish that their clinical status has not changed from their initial evaluation. The patient will be prepared for surgery with dexamethasone and an anticonvulant, and taken to the operating room for stereotactic surgery. An MRI compatible Cosman-Roberts-Wells (CRW) stereotactic frame (Radionics, Inc., Burlington, MA) will be placed on the patient's head under antiseptic technique using local anesthesia. The patient will then be taken to the MRI scanner where the tumor will be localized by MRI scan with gadopentate dimeglumine contrast.

The tumor measurements from the MRI scan will be analyzed using Stereoplan™ software program (Radionics, Inc.). A three dimensional representation of the tumor will be constructed identifying the points of injection and depths of injection to be utilized to provide complete infiltration of tumor. The patient will be returned to the operating room, placed under general anesthesia, and the CRW arc will be attached. The tumor will be stereotactically infiltrated with H5.010RSV*TK*. The virus will be injected slowly so as to prevent the virus suspension from extravasating along the 22 gauge needle injection tract. We anticipate that we will be injecting tumors varying in size from 20 to 50 cm^3 with an average of 35 cm^3 in volume. In the two phase I studies we initially plan to treat a total of 24 patients. In each study (resectable and unresectable tumors) there will be three patients at each dose of virus (10^8, 10^9, 10^{10}, and 10^{11} pfu). The total virus volume will be 1.0 ml. Postoperatively, the patient will be treated with prophylactic antibiotics (either cefazolin or vancomycin). The patient will remain in a private room on the surgical intensive care unit under continuous observation for signs of increased intracranial pressure. Serum electrolytes, urine specific gravity and neurologic status will be monitored. Examinations will be performed daily, or more frequently, by a neurosurgeon and a neurologist who are investigators. On the second postoperative day, intravenous ganciclovir will be started at a dose of 5 mg/kg IV over one hour, every 12 hours, and continued for 14 days, or until the second surgery (in the case of patients with resectable lesions). When clinically stable, the patient will be transferred to the GCRC. This is expected to be 1 to 2 days postoperatively. Standard MRI scans will be repeated within 48 hours and volumetric MRI scans at 7 days after surgery, or earlier if a significant change in size of tumor is suspected by the standard MRI. Seven days after stereotactic surgery, the patients in the phase IA study will undergo open craniotomy. This will provide debulking of a recurrent tumor which is the standard procedure for a patient with good performance status and tumor in a resectable location, and tissue to be analyzed for effects of gene therapy. At the time of the second surgery, the tumor bed will be infiltrated with the same dose of H5.010RSV*TK* as used in the patient's first treatment. GCV will be given for 14 days following tumor resection.

III.C Evaluation for Safety

III.C.1 Neurologic and Surgical Risk

Patients may be at risk for the usual complications of brain surgery which include hemorrhage, edema, permanent or temporary neurological damage or infection. Preoperative corticosteroids will be given to decrease these risks. Careful neuralgic examination will be performed daily while patients are hospitalized (for two weeks after the surgical injection of the virus), and a record will be kept of the neuralgic findings. The physician will assess for level of consciousness, signs of increased intracranial pressure, seizure activity and focal signs. The MRI scan will be repeated once weekly for two weeks, and then monthly. In case of any unexpected neurologic changes that persist, the MRI will be performed more frequently. The MRI will be used to assess for increased edema and postoperative hemorrhage. Increased intracranial pressure will be treated with higher doses of corticosteroid or Mannitol. Prophylactic antibiotics will be given to reduce the risk of wound infection, which may be greater than average because of the previous surgery and scalp irradiation these patients have received.

III.C.2 Virus Infection

Patients will be assessed for evidence of virus dissemination, with chest x-rays, examination of spinal fluid in patients where it is deemed safe to perform a lumbar puncture, and MRI will be analyzed for signs of encephalitis and meningitis. Biopsies performed after viral injection in patients with resectable tumor will be submitted for viral culture and histologic examination for encephalitis.

III.C.3 Ganciclovir Toxicity

The major toxicity is expected to be myelosuppression. Complete blood counts will be performed every 2 to 3 days after adenovirus treatment. Dose adjustment for bone marrow toxicity will be as follows: absolute granulocyte count (polymorphonuclear leukocytes and bands) $<1000/mm^3$ or platelet count $<100{,}000/mm^3$; reduce dose to 75%; absolute granulocyte count $< 500/mm^3$ or platelet count $<50{,}000/mm^3$: discontinue ganciclovir, and resume the drug at 50% of the original dose when the granulocyte count is >500 and platelets $>50{,}000$. Less frequent side effects of ganciclovir include headache, venous irritation with pain or redness, renal insufficiency, infertility, birth defects, fever, rash, and liver function abnormalities. Patients will be assessed for headache and fever and treated symptomatically but the dose will not be adjusted. Venous irritation will be avoided by the use of central venous catheters (such as a Hickman port or Portacath) in patients who have poor venous access. Renal function tests (BUN, Creatinine) will be performed weekly. If the level of creatinine rises to >2, the ganciclovir will be discontinued until the creatinine falls below 2 and then resumed at 75% of the original dose. Women of child bearing age will be requested to use effective contraceptives during and for 3 months

after the treatment. Ganciclovir will be discontinued if grade 3 or 4 skin or hepatic toxicity occurs.

III.C.4 Dose Escalation

Patients will be enrolled in phase IA and phase IB studies in groups of three. Each group will be followed for a minimum of 30 days before enrolling patients to the next higher dose within each study. If one or more patients in a group develop dose limiting toxicity, then three subsequent patients will be enrolled at a one log lower dose. If these patients are successfully treated without dose limiting toxicity, then treatment at the next higher dose will be done in three additional patients. If none of these patients experience dose limiting toxicity, the planned dose escalations will continue. If any develop dose limiting toxicity, the study will stop enrolling patients.

III.C.5 Evaluation for Dissemination of Virus

Evidence of dissemination of the adenovirus vector will be systematically sought, although we believe that this is an unlikely event. In the cystic fibrosis gene therapy trial, up to 10^9 pfu of adenovirus was instilled into the lungs of patients. No dissemination was detected. Since installation into the brain represents a more contained administration of the virus, we feel that dissemination is less likely. If it dose occur, the probable routes of initial spread will be via the cerebrospinal fluid (CSF) and via the blood stream. Entry into the CSF can occur via direct contact with the virally infected tumor, and entry into blood vessels can occur through damaged tumor capillaries. CSF, blood, urine, stool and respiratory samples will all be analyses for the presence of the viral vector. If detected, the findings will be reported to the FDA.

III.C.6 Dose Limiting Toxicity: Definition

Dose limiting toxicity (DLT) will be defined as (1) neurologic toxicity of grade 3 or 4, (2) clinical or MRI manifestations of generalized encephalitis or meningitis, (3) increase in edema or mass effect by MRI scan, associated with development of herniation, or decrease in level of consciousness to Glasgow Coma Scale of 10, despite appropriate measures (e.g., mannitol, corticosteroids). However, if these changes occur more than 2 weeks post-operatively and are associated with an MRI appearance consistent with tumor growth, this will not be considered dose limiting toxicity, (4) any other grade 3 or 4 toxicity in other organ systems which is not attributable to ganciclovir and which persists despite discontinuation of ganciclovir. Any patient experiencing DLT will be removed from the study.

In the operating room, universal precautions will be observed. All instruments will be soaked in bleach after the procedure, and disposable instruments and linens will sealed in autoclave bags and removed from the operating room. No pregnant medical personnel will be permitted to participate in the surgical procedure.

III.D Evaluation for Biological Efficacy

The primary end points will be radiographic and histologic evidence for tumor regression. MRI and PET studies will be used to evaluate tumor response. In those patients undergoing palliative resection, tissue from the tumor bed will be removed for histological examination for evidence of tumor, as well as possible adverse effects of therapy such as edema, demyelinization, inflammatory reaction. These findings will be correlated with the MRI and PET scans.

III.D.1 Radiological Evaluation

We will adopt a comprehensive approach to magnetic resonance imaging of these brain tumors. Our evaluation can be divided into two broad goals, the first will be "classical" anatomic issues, the second will be attempts to improve characterization of tissue changes associated with primary tumors and therapy. The major anatomic goals will be to define the extent of the tumor and associated abnormalities. These are: (1) Volume of enhancing tissue as measured on "T1-weighted" volumetric gradient-echo image sets obtained before and after administration of gadopentetate dimeglumine contrast material. (2) Volume of "edema" detected on Fast Spin-Echo "T2-weighted" images, recognizing that this will include portions of the neoplasm, white matter infiltrated by neoplastic cells and vasogenic edema located beyond the confines of the tumor. (3) Presence and severity of mass effect will be evaluated using linear measurements on the T1- and T2-weighted images. For obvious reasons, these results will be expected to be highly correlated with 1 and 2, above. (4) Local cerebral blood volume measurements will be determined using dynamic susceptibility-contrast imaging following a bolus injection of contrast material. MR-determined blood volume has been reported to correlate with tumor vascularity, mitotic activity and histologic grade [43]. Thus, these measurements may be helpful in characterizing treatment response of residual tumor. These images are obtained using an echo-planar sequence performed during the first 60 seconds after rapid intravenous infusion of gadopentetate dimeglumine contrast material. (5) Magnetization transfer contrast has proven useful in characterizing a variety of white matter abnormalities [48]. For the purpose of monitoring the effectiveness of therapy, we will investigate its ability to distinguish neoplastic tissue from encephalomalacia, radiation necrosis and regions containing edema with relatively few tumor cells.

III.D.2 Positron Emission Tomography (PET) Evaluation

Regional cerebral glucose metabolism will be measured using the FDG technique of Reivich et al. [49]. A venous catheter will be inserted into an antecubital vein of one arm for the administration of FDG and second venous catheter will be inserted into the opposite hand for "arteriolized" blood sampling. FDG will then be administered as a bolus (114 mci/kg), 2 ml blood samples will be obtained every 15 seconds for the first minute, then a less frequent intervals for the duration of the study. The blood samples will be centrifuged and aliquots of plasma analyzed for 18F activity and glucose concentration. Forty minutes after the administration of FDG, the patient will be positioned into the PENN PET scanner (UGM Medical Systems, Inc.). The PET scans will be acquired parallel to the canthomeal line and will include the entire brain. The total imaging time will be 30 minutes which in our experience is tolerated well by most patients. Regional glucose metabolic rates will be calculated us-

ing the operational equation, derived by Sokoloff et al. [50] and as modified by Huang et al. [49,51] will be used in the calculation of the metabolic rates.

The Penn-PET scanner is unique in its ability to sample continuously in the transverse and axial directions. Its large field of view in the axial axis (12.8 × 9.5 cm) permits imaging of the entire brain in one data acquisition session. The spatial resolution of the system in 5.5 mm in all three planes, specifications that are unique to this instrument. This high resolution reduces partial volume effects, an important consideration with small or irregular shaped tumors. Slice thickness can be varied with this scanner and we will use 2 mm sampling in the z-axis for this study. The equally good spatial resolution in all planes allows the data to be treated as a volume and resliced along any planes for subsequent MRI and PET image superimposition and data analysis.

FDG PET studies will be analyzed using PETVIEW, a SUN workstation-based program developed at PENN with UGM Medical Systems, Inc. This analysis system acquires data directly from a UGM PENN-PET SUN workstation and permits superimposition of PET and MRI images. MRI and PET images are constructed in the transverse plane and stacked to form a volume image. Each section is then resliced parallel to the anterior commissure-posterior commissure line using the OBLIQUE software module of PETVIEW. The slice containing caudate nucleus on PET and MRI is used as visual guide confirm the accuracy of image overlay and for positioning in the z-axis.

We intend to use both qualititative (visual interpretation) as well as quantitative approach to determine the metabolic activity of the tumoral sites. Qualitative assessment will use the following grading system for FDG tumor uptake: (1) no FDG tumor uptake; (2) less than surrounding brain; (3) indistinguishable from adjacent brain; (4) slightly to moderately increased compared with adjacent brain; (5) markedly increased compared with adjacent brain. Quantitative assessment will include measurements of regional glucose metabolic rates and calculated ratios of tumor:normal brain [43] glucose utilization. Absolute glucose metabolic rates vary widely in brain tumors and their correlation with histology or clinical tumor to that of (a) whole brain, (b) contralateral white matter or (c) cerebellar hemisphere, are closely correlated with histology. For this study, brain tumor boundaries will be identified on MR images, then superimposed on co-planner PET slices. Average, peak and decade profile (i.e., 10% bins) glucose metabolic rate values will be calculated for the tumor and for centrum semiovale white matter in the hemisphere contralateral to tumor.

III.D.3 Pathologic Evaluation

Tumor specimens from patients undergoing open resections will be received in Surgical Pathology of the University of Pennsylvania. Some of tissue will be fixed in formalin and embedded in paraffin and histologic sections will be stained with hematoxylin and eosin. Portions of the tumor will be preserved in OCT for immunohistochemical staining, and frozen for PCR analysis of adenovirus DNA. Immunohistochemical stains for glial fibrillary acidic protein, neurofilament protein and factor VIII, adenovirus proteins and HSV*tk* protein will be performed. Tumors will be classified according to the W.H.O. classification for brain tumors. An estimate of the percentage of tumor composed of proliferating vascular tissue will be made. PCR for DNA and RNA will be utilized to detect recombinant virus in the surgical pathology brain tumor specimens. A postmortem examination will be requested on all protocol patients. Gross examination and histologic analysis of systemic organs will be performed. Analysis of the brain will include an estimate of volume of tumor. Sections of residual tumor and normal brain will be snap frozen in liquid nitrogen and stored at −80°C. Portions of residual tumor and surrounding brain will be submitted for histologic processing. In addition to the tumor, sections will be taken for histologic analysis from cortex and white matter of the frontal, parietal, temporal and occipital lobes, basal ganglia, thalamus, hippocampus, cerebellum, pineal, cervical, thoracic, lumbar and sacral levels of the spinal cord and the pituitary gland. In situ hybridization for recombinant virus will be performed.

III.E Evaluation for Immunological Response

Serum and cerebrospinal fluid will be collected pre and post therapy for detection of anti-adenovirus antibodies by western blot and by neutralizing antibody assay. The patient's humoral immune response to adenovirus will be studied by determining titers to adenovirus at various times during the protocol. When autologous tumor tissue is available (tumor obtained prior to the gene therapy), cell mediated immunity will be studied to see if the patient's lymphocytes will proliferate in response to tumor cells, to adenovirus, or to tumor cells that have been transduced with the virus in vitro. Similarly, cytotoxic T-cell responses to the patient's tumor cells will be determined using lymphocytes obtained both pre and post gene therapy for this purpose. The interpretation of lymphocyte testing may be confounded by a clinical requirement for the use of corticosteroids. These tests may provide evidence for the development of an immune response to following adenovirus transduction and will play an important role in the design of future adenovirus vector clinical trials. The immune response to the vector may limit the efficacy of serial treatments, may lessen the duration of transgene expression, or may contribute to local, dose limiting, inflammation [52]. These problems may be overcome by the use of newer adenoviral vectors that are currently under development [53].

III.F Ethics Advisory Board

An Ethics Advisory Board within the Institute for Human Gene Therapy will undertake the development of guidelines and recommendations with respect to all areas of patient participation in gene therapy research.

IV. Isolation and Production of H5.010RSV*TK* Adenovirus

IV.A Construction of recombinant adenoviral vector-H5.010RSVTK

The strategy for constructing and producing this recombinant adenovirus is summarized as follows. Step 1. Construction of pAdBgIII. The purpose of this step was to construct a plas-

mid containing the 5′ portion of Ad 5 with deletion of E1 sequences and a unique cloning site. The plasmid pEHX-L3 contains sequences from Ad5 spanning map units 1 to 16.1. pEHX-L3 was digested with EcoRI and BglI and a 5.2 kb fragment isolated, which contains the adenoviral sequences from map units 9.2–16.1 and the plasmid backbone (derived from pAT 153). The adenoviral sequences from map unit 0–1 which contains the 5′ inverted terminal repeat, origin of replication and encapsidation signal were amplified from the original pEHX-L3 using PCR to insert a Nhe I site immediately downstream of the EcoRI site, and a BglII site at the 3′ end. This PCR fragment and the EcoRI/Bgl II 5.2 kb fragment were ligated to produce the plasmid pAdBglII.

Step 2. Construction of pAd.RSV*TK*. The RSV promoter, the HSV *TK* gene and the SV40 polyadenylation signal were cloned into the BglII site of AdBglII. The source of the RSV promoter and the SV40 polyadenylation signal was the commercially available plasmid pREP8 (InVitrogen). pREP8 contains the RSV promoter and SV40 polyadenylation signal flanking a small multiple cloning site. pREP8 was cut with Not I and Kpn I to remove the NheI site. The ends were blunted, ligated to Not I linkers and excess linkers cleaved. The resultant plasmid was ligated (closed). The RSV-MCS-SV40 poly A was removed from this plasmid by digesting with Nru I and Xba I. This MCS in this cassette contains a unique Not I, Bam HI and Xho I site. Ends were blunted and Bgl II linkers added. This fragment was then cloned into pAdBglII and designated pAdRSV4. The HSV*tk* gene was isolated from the plasmid pL(X)RNL.HSV*tk* (gift of XO Breakfield). pL(X)RNL.HSV*tk* was cut with Bgl II and Bam HI to remove the HSV*TK* fragment. The Bgl II digestion results in removal of a large portion of the 5′ untranslated region. The fragment was gel isolated and cloned into Bam HI cut pAdRSV4. Recombinant plasmids in which the HSV*tk* gene was correctly inserted were amplified and designated pAdRSV*tk* (this adenovirus has been renamed, H5.010RSV*TK*). The plasmid DNA was linearized with Nhe I prior to generation of adenoviral recombinants.

*IV.B Generation of Recombinant H5.010RSV*TK *Virus*

The recombinant *tk* adenovirus was constructed from the plasmid pAdRSV*tk* and modified adenovirus type 5 (Ad 5), *sub 360*, in which a small portion of the E3 gene is deleted. pAdRSV*tk* was linearized by Nhe I and co-transfected with the large fragment of XbaI/Cla I cut sub 360 into 293 cells (a human kidney cell line containing a functional E1a gene that provides a trans-acting E1a protein) to allow homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infectious virions and formation of plaques. Individual plaques were isolated and amplified in 293 cells, viral DNA was isolated, and recombinant adenoviral plaques containing the human *tk* cDNA were identified by restriction cleavage and Southern blot analysis. Three of the positive plaques were plaque-purified a second time and re-scored by restriction digest and southern analysis. One of the positive plaques from the second purification was amplified, purified by cesium chloride gradient ultracentrifugation, desalted, and stored in the −80°C freezer in 10% glycerol in PBS. Viral stocks were then tested for the ability to confer ganciclovir sensitivity to rat glioblastoma cell lines.

*IV.C Sequence Analysis of Recombinant Virus, H5:010RSV*TK

H5.010RSV*TK* viral DNA was isolated from the purified viral preparation obtained after two rounds of plaque purification. Selected areas of the viral genome were sequenced by an approved FDA facility in compliance with GLP (Good Laboratory Procedures).

Two areas of the genome subjected to complete sequence analysis include 1) the 5′ end of the genome spanning the 5′ ITR, the entire minigene cassette including RSV promoter, the *TK* cDNA, and 2) the region surrounding the E3 deletion. The regions sequenced were subcloned as overlapping restriction fragments into pBluescript II (Stratagene) and pGem5Zf (Promega). Nested deletion clones were generated in both directions for each of the subclones using a modified exoIII S1 nuclease procedure. These deletion clones will be size selected to provide complete coverage of each strand and sequenced using the dideoxynucleotide termination procedure. Internal sequencing primers were synthesized and used to close gaps between contigs and to fill in any single-stranded regions.

IV.D Strategy for Characterization of Clinical Grade Adenovirus—Quality Assurance and Control

A four-stage test program has been designed to assess the master cell bank, the seed lot, the product intermediate (cell lysate) and the purified product (virus). The 293 cells used to produce the adenovirus will be characterized prior to infection for possible microbial, adventitious viral and select specific human viral contaminants. Testing of the adenovirus preparation used to infect the cells will include assays for microbial contaminants and adventitious virus. After expansion of the infected cells, the cell lysate will be evaluated for microbial contaminants. Product testing of the purified product for endotoxin, microbial contaminants, extraneous toxins and infectious adenovirus completes the test battery. Each step will be carefully tested for the presence of wild type adenovirus. The majority of tests will be performed by Microbiological Associates Inc. (MAI), an independent laboratory that has provided contract research and safety assessment for the pharmaceutical industry.

Similar to the generation of recombinant retroviral gene therapy reagents, adenoviral reagents have been constructed to provide "cassette" oriented approaches to their production. A recombinant adenoviral vector was constructed from a modified adenovirus type 5 in which the minigene of interest is inserted in place of E1a and E1b. This vector is cotransfected along with the large fragment of the enzyme-restricted Ad5 DNA into 293 cells. 293 cells are a human kidney cell line that contains a functional E1a gene and provides a trans-acting E1a protein in order to allow for homologous infectious virions and formation of plaques. A Master Cell Bank (MCB) of 293 cells has been previously established and evaluated for performance, in terms of production of recombinant adenoviruses and for the absence of other pathogenic contaminants. Individual plaques are isolated and functional DNA would be identified by restriction cleavage and Southern blot analysis. The MCB is then infected with the crude viral lysates and plaque-purified a second time in order to generate a seed lot using 293 cells. The purified seed lot lysate is subjected to safety testing. The MCB is then plated

again and infected with the certified viral seed lot. Lysates are harvested from the infected cells and virus is purified from the lysate and cryopreserved. Individual production lots are extensively evaluated. The purified adenoviral DNA is then subjected to sequencing.

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University of Pennsylvania Informed Consent

Treatment of advanced CNS malignancy with recombinant adenovirus: Phase 1A

CONSENT TO ACT AS A SUBJECT IN AN INVESTIGATIONAL STUDY

1) Title of research project:
Treatment of advanced CNS malignancy with recombinant adenovirus: Phase 1A.
2) Investigators: Jane B. Alavi, Abass Alavi, Stephen Eck, Joan Mollman, Kevin Judy, David Hackney, Peter Phillips (Telephone number of Dr. Alavi: 662-6319 or 662-6059; telephone number of Dr. Eck: 734-8226)
3) Purpose: The purpose of this study is to determine the safety of a new form of brain cancer therapy. In this study a recombinant (laboratory-produced) adenovirus will be directly injected into your brain cancer. (An adenovirus is a common virus which often causes infections of the nose and windpipe and lungs). You will then receive a drug called ganciclovir. The primary purpose of this study is to determine if this type of treatment can be safely done in humans. The virus has been especially designed to carry a gene, herpes simplex thymidine kinase (HSV-TK). It is also altered to make it difficult for it to multiply. The study will also investigate whether this gene (a gene is made of DNA) will enter brain tumor cells and cause the tumor cells to be killed by ganciclovir.

You have been asked to participate because you have a brain tumor which failed to respond to conventional therapy.
4) Description of the project:
1) Pre-gene therapy evaluation. You will be evaluated to determine if you are a suitable subject to participate in the gene therapy study. This will involve a full medical history and physical examination, blood tests, MRI scan of the brain and chest x-ray. Approximately 30 ml (2 tbs.) of blood will be taken from an arm vein. A pregnancy test will be performed if you are a woman with child bearing potential.

2) Gene therapy study. You will be admitted to the Clinical Research Center or the Neurosurgery Service of the Hospital of the University of Pennsylvania. A positron emission tomography scan (PET scan) of the brain will be performed. For this scan, you will lie on a comfortable reclining chair in the Nuclear Medicine Section of the hospital, and scans (pictures) will be taken of your brain. Your head will not be enclosed during this procedure. Prior to the scans, a needle will be placed in the radial artery (at the wrist) for samples of blood. Another needle will be placed in an arm vein (in the hand or elbow area) and used for injection of a radioactive form of sugar, F-18 deoxyglucose. The purpose of the PET scan is to determine if your brain tumor uses sugar, as many malignant tumors do. The PET scan will be repeated at 2 weeks and 4 weeks after gene therapy, to see if the treatment has changed the characteristics of the tumor.

On the second hospital day you will be taken to the operating room. Under local anesthesia, a stereotactic frame will be placed on the outside of your head. This is a large ring attached to the scalp temporarily, to permit precise measurements of the tumor location. You will be transported to the MRI scanner for an MRI scan of the brain, then back to the operating room. General anesthesia will then be administered and the neurosurgeon will perform surgery by stereotactic technique. In other words, the brain will be entered through small holes rather than through a large incision in the skull. The surgeon will inject into the tumor a small amount of the recombinant adenovirus, containing the herpes simplex thymidine kinase (TK) gene. After the surgery, you will be taken to the Intensive Care Unit for routine post operative care. You will be transferred back to the Clinical Research Center (CRC) in 1 or 2 days. The stereotactic frame will be removed after surgery.

Two days after the surgery, you will begin to receive the drug ganciclovir in the vein, twice a day, for seven days. You will have an MRI scan of the brain on the second and seventh post operative days.

Approximately 7 days after the stereotactic surgery, you will have a second operation. This will be a standard neurosurgical operation under general anesthesia, to remove as much of the brain tumor as is safe and possible. After the tumor is removed, the surgeon will inject another dose of the same adenovirus into the area of remaining tumor. After this operation you will again be treated in the Intensive Care Unit or the CRC, with twice a day injections of ganciclovir for 14 more days. MRI scans will be performed 2 and 14 days after surgery. You will be discharged from the hospital after 14 days, or whenever your condition is considered medically stable.

Lumbar puncture may be performed one or two times after the injection of the virus, to sample spinal fluid.

You will need long term follow up. Because gene therapy is a very new form of treatment, the long term effects are not known. To gain the most information and to provide the greatest safety, periodic evaluations will be needed. Your agreement to participate in this study means that you recognize the need for prolonged relationship with the study and appreciate the need for continued evaluations. This will entail monthly evaluations that include history, physical examination, MRI scan and routine blood tests for one year, and less frequent evaluations for as long as you live.

Consent for autopsy. To fully evaluate the effects and safety of gene therapy, it will be necessary to obtain as much information as possible. In the eventual occurrence of your death, evaluation of your brain and other organs will be a very valuable method to see the full effects of gene therapy. Therefore your participation in this study means that you agree to an autopsy. This agreement must also be known by your next of kin so that person can follow with your wishes.

5) Risks and discomforts of the research.

Blood tests: You may develop a bruise or tenderness at the site of needle punctures.

Ganciclovir: This is a drug used for certain virus infections. The most common side effects are headache, irritation of the veins, lowering the white blood cell count (which could lead to infection), lowering of the platelet count (which could lead to bruising or bleeding). Rarely, kidney damage can occur. You will be monitored with frequent blood tests to look for these side effects, and the dose of ganciclovir will be decreased or stopped if serious adverse effects occur.

Lumbar puncture: This can lead to pain, bruising or bleeding in the back where the needle is placed.

Surgery: You will be asked to sign a separate consent form for the neurosurgery. The potential risks include paralysis, stroke, difficulty with speech or vision that may be temporary or permanent, bleeding into the brain, infection, and possible death. The study requires that you have two brain operations, whereas normal treatment would involve only one operation.

PET scans: The arterial and venous catheters (tubes) could cause some bleeding at the site of the injection. The tube could become disconnected causing blood loss or the artery or vein could become clotted. This could cause circulation problems in the hand making it cold and painful. Pieces of the tube or clot could break off and lodge elsewhere requiring treatment. These complications are extremely rare. The PET scan utilizes radioactive material and therefore you will receive a radiation dose. The radiation dose you receive is within the limits permitted by the FDA for human research volunteers. While the radioactive material is in your body it is not hazardous to persons near you. Also it will have largely disappeared within 6 hours. These studies will not be performed on pregnant women. A blood pregnancy test will be done on any woman of child bearing potential.

In this research study, you will be asked to have more frequent scans and tests than is normal for patients undergoing brain tumor treatment.

Complications caused by the virus. The virus that is being used has been made so that it cannot reproduce by itself. There is a very slight possibility that something may happen during the study that will allow the virus to multiply. The effects of this event are unknown. However your immune system will likely kill the virus after it has been there for several days to weeks. It is possible that the virus could spread from your body to your surroundings. The virus has been altered so that it can not reproduce by itself. If however, something should happen that the virus does spread and reproduce, it would likely behave no differently from similar types of viruses that are already in the environment around us. However, if there are signs that virus is being spread, you may be asked to remain in the hospital longer than 2 weeks for further monitoring. The type of virus used in these

studies is not the type that inserts its genes into the patient's own genes. The insertion into normal genes is thought to be a cause of cancer in very rare instances. Because insertion of the virus gene into your genes is highly unlikely, the chance that the virus will cause cancer is very small. There is a remote possibility that the virus could change genes in the body's eggs or sperm. Therefore patients who are pregnant are not permitted to enter this study, and all patients in this study are advised to take precautions not to bear children in the next few months following treatment.

Withdrawing from the study. If you withdraw from the study after the gene is placed into your brain, it will not be possible to evaluate fully the safety of gene therapy. Should complications occur, it might not be possible to detect them promptly and institute therapy.

Loss of privacy. Gene therapy is very interesting to the general public. Although every effort will be made to maintain your privacy, it is possible that the news media or other individuals might learn of your identity and spread this information, even if you do not want this to happen.

Participation in multiple studies may be hazardous to you. If you are already participating in another research study, please inform us fully. You should not participate in multiple studies, unless you and the investigators agree that your health and the outcome of the study will not be jeopardized.

6) Expected benefits. You may not benefit in any way from this research study. Similar studies in laboratory animals have shown that this virus in combination with ganciclovir can cause tumors to decrease in size. If the treatment does result in killing of tumor cells, the tumor may become smaller for a period time, but it is not known if this will occur or for how long any benefit will last. Since the purpose of this study is to determine the safety of new techniques, we do not expect that you will benefit from participating, although knowledge may be gained that may benefit others.

7) Alternative procedures. Alternative treatments for brain tumors include standard surgery, chemotherapy or sometimes implantations of radioactive sources into the brain.

8) Costs to subject or insurance carrier resulting from participation in the study. There will be no cost to you for your participation in the study. Any costs resulting from care you require as part of the study or because of complications from the study will be provided through the Clinical Research Center of the University of Pennsylvania.

9) Payments. You will not be paid for participating in this study.

10) Confidentiality. You will not be identified in any reports on this study. The records will be kept confidential to the extent provided by federal, state and local law. The Federal Food and Drug Administration may inspect the records of this investigation. The extreme amount of public interest in gene therapy may cause members of the media to try to find out who you are. If they succeed, you might be subject to frequent requests for interviews and your privacy may be reduced. Every effort will be made to protect your privacy but this may not be possible.

11) Management of physical injury. You will remain in the hospital for 2 weeks after the surgery. If problems or questions arise when you are not in the hospital you should contact Dr. Jane Alavi at 662-6319 or 662-6059 (24 hour number) or Dr. Stephen Eck (734-8226, a 24 hour number). In the event of a physical injury which may result from research procedures, the University will provide medical treatment. Additional medical treatment will be provided in accordance with the determination by the University of its responsibility to provide such treatment. However, the University does not provide compensation to a person who is injured while participating as a subject in research.

12) Availability of further information. If you wish further information regarding your rights as a research subject, you may contact the Office of Research Administration at the University of Pennsylvania by telephoning (215) 898-7293. If significant new knowledge is obtained during the course of this research study which may relate to your willingness to continue participation, you will be informed of this knowledge. To find out more about any aspect of the study including your rights, you may contact the investigators.

13) Voluntary nature of participation. Your participation in this project is voluntary. You may refuse to participate in or withdraw from the study at any time without penalty or loss of benefits to which you may otherwise be entitled. In case you decide to withdraw from the study you may suffer the following consequences. 1) Loss of the ability to monitor for side effects of gene therapy. 2) Failure to find out if there are any unforeseen risks from gene therapy that may not be obvious without repeated evaluation.

14) Documentation of consent. One copy of this document will be kept together with our research records on this study. A second copy will be placed in your hospital record. A third copy will be given to you to keep.

15) Consent of the subject. I have read the information given above. I understand the meaning of the information. The investigator has satisfactorily answered my questions concerning the study. I hereby consent to participate in the study. I will also have my next of kin sign a provisional consent to an autopsy to be used when I eventually die.

16) Names and signatures of consenting person and witnesses.

______________________________ ______________________________

Subject's signature Date

______________________________ ______________________________

Witness' signature Date

______________________________ ______________________________

Investigator's signature Date

CONSENT TO ACT AS A SUBJECT IN AN INVESTIGATIONAL STUDY

1) Title of research project:
Treatment of advanced CNS malignancy with recombinant adenovirus: Phase 1B.

2) Investigators: Jane B. Alavi, Abass Alavi, Stephen Eck, Joan Mollman, Kevin Judy, David Hackney, Peter Phillips (Telephone number of Dr. Alavi: 662-6319 or 662-6059; telephone number of Dr. Eck: 734-8226)

3) Purpose: The purpose of this study is to determine the safety of a new form of brain cancer therapy. In this study a recombinant (laboratory-produced) adenovirus will be directly injected into your brain cancer. (An adenovirus is a common virus which often causes infections of the nose and windpipe and lungs). You will then receive a drug called ganciclovir. The primary purpose of this study is to determine if this type of treatment can be safely done in humans. The virus has been especially designed to carry a gene, herpes simplex thymidine kinase (HS-TK). It is also altered to make it difficult for it to multiply. The study will also investigate whether this gene (a gene is made of DNA) will enter brain tumor cells and cause the tumor cells to be killed by ganciclovir.

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Two days after the surgery, you will begin to receive the drug ganciclovir in the vein, twice a day, for fourteen (14) days. You will have an MRI scan of the brain on the second, seventh and fourteenth post operative days. You will be discharged from the hospital after 14 days, or whenever your condition is considered medically stable.

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16) Names and signatures of consenting person and witnesses.

Subject's signature	Date
Witness' signature	Date
Investigator's signature	Date